Activation of the *hTERT* expression in squamous cell cervical carcinoma is not associated with gene amplification

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Abstract. The *hTERT* gene encodes the telomerase catalytic subunit that plays a key role in cancer cell immortalization. Earlier, *hTERT* amplification was detected in squamous cell cervical carcinomas (SCC), however possible relations between elevated *hTERT* mRNA level and gene amplification was not studied. Here, we compared the *hTERT* expression and copy number in the same tumors by quantitative real-time PCR. The *hTERT* DNA copy number was virtually unchanged in all 33 studied tumors, when compared to normal tissues. This result was confirmed using two reference genes ß-actin and ß-D-glucuronidase. Nevertheless, the activation of *hTERT* expression was found in 80% of cases (37/46, $p<0.001$). There was no correlation between the degree of mRNA increase and the tumor size and/or presence of metastases. No *hTERT* gene expression was observed in 20% of cases (9/46), while the control *GADPH* expression was unchanged. The detected elevation of the *hTERT* mRNA level was found using primers specific to functionally active full-length isoform of mRNA. Similar results were obtained with SCC cell lines carrying human papilloma virus (HPV) genomes. We conclude that frequent activation of *hTERT* expression in SCC is not associated with gene amplification.

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Abbreviations: ACTB, beta-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GUSB*, beta-D-glucuronidase; HPV, human papilloma virus; *hTERT*, gene coding catalytic subunit of telomerase; SCC, squamous cell cervical carcinomas; qPCR, quantitative real-time PCR

Key words: telomerase, *hTERT* mRNA level, *hTERT* DNA copy number, squamous cell cervical carcinoma, quantitative real-time PCR

Introduction

Telomerase plays a key role in controlling cell proliferation activity. This enzyme was detected in most immortalized tumor cells and stem mammalian cells (1). Telomerase is a ribonucleoprotein complex, composed of an RNA component (hTR), catalytic subunit (hTERT) that exhibits the reverse transcriptase activity, and a number of accessory proteins. Telomerase catalyses telomere elongation and maintains the proper telomere size. The hTR is constitutively expressed in all cells (2) in contrast to the hTERT that is not expressed in differentiated cells.

The *hTERT* gene is localized on chromosome 5p15.33 and composed of 16 exons. Only full-length isoform of *hTERT* mRNA encodes a catalytically active telomerase. Mechanisms controlling the level of this mRNA isoform probably include transcriptional regulation (e.g. promoter demethylation, (in)activation due to transcription factors binding), alternative splicing and gene amplification. The *hTERT* gene promoter contains binding sites for repressors (p53, Mad1, etc.) and activators of expression (c-Myc, Sp1) (3). The *hTERT* gene amplification was found in different primary tumors and cell lines. In squamous cell cervical carcinomas (SCC), an increase in *hTERT* copy number was found in 24% of cases. It correlated significantly with higher levels of *hTERT* protein expression (4,5).

SCC is characterized by well defined clinical stages (from several forms of intraepithelial dysplasia to invasive carcinomas); and by the presence of integrated or episomal forms of human papilloma virus (HPV) DNA from so-called high risk group (HPV types 16, 18 and related). Recent qPCR studies showed that *hTERT* mRNA level increased even in precancerous lesions (6). However, the role of *hTERT* activation at early stages of SCC remains unclear. Gene amplification was discussed as one of the possible mechanisms of *hTERT* activation (4,5). Here we quantitatively measured *hTERT* gene copy number and mRNA level in the same SCC specimens and found that elevation of *hTERT* mRNA level is not associated with gene amplification.

Materials and methods

Specimens. SCC and adjacent morphologically normal tissues were obtained from patients after surgery in accordance

with the rules issued by the Ethics Committee of Blokhin Cancer Research Center, Moscow, Russia. The samples were immediately frozen and stored in liquid nitrogen. All tumors were classified histopathologically and characterized according to the International System of Clinico-Morphological Classification of Tumors (TNM). The HPV genome from the 'high-risk' group (types 16 and/or 18) was present in all carcinomas as described earlier (7).

Cell culture. Five SCC cell cultures were obtained from American Type Culture Collection: four HPV-containing lines (HeLa with multiple copies of HPV18 and CaSki, SiHa, and C4-1 cells with different copy numbers of HPV16) and one HPV-free line (C33a).

DNA and RNA extraction. Total DNAs and RNAs were extracted and isolated as described earlier (8). DNAs and RNAs were analysed by electrophoresis in 2% agarose and quantified using spectrophotometer (Nanodrop Technologies Inc).

DNA samples from 33 SCC tissues and 17 samples of adjacent morphologically normal tissues were analyzed. Forty-six mRNA samples from SCC tissues and 20 samples from adjacent morphologically normal tissue were also studied. All RNA samples were treated with DNase I and cDNA was synthesized using MMLV reverse transcriptase and random hexamers according to standard manufacturer's protocol (Fermentas, Lithuania). Thirty-three DNA and mRNA samples were obtained from the same SCC tissues.

Primer design. The primers and probes were designed using the Primer Design program (EIMB RAS, Russia, www.imb.ac.ru/~nixie/PD/PD.rar).

Set 1: Primers specific to exon 2 of *hTERT* mRNA and common for genomic *hTERT* DNA and all known mRNA isoforms (genomic positions 1606-1623, forward, and 1711-1729, reverse). Forward, reverse primers and probe were as follows: 5'-AACGAACGCCGCTTCCTC-3'; 5'-GCTCC TGCGCAGCCAAG-3'; 5'-CAGTCCCGCACGCTCATCTT CCACGT-3', the amplicon length is 142 bp.

Set 2: Primers specific to full-length *hTERT* isoform 1 only. Forward primer crossed the boundary of exon 5 and exon 6 (genomic region 2174-2198). Reverse primer was specific to isoform 1 and 2, it was located in exon 7 (genomic region 2353-2374). Forward, reverse primers and probe for *hTERT* gene were as follows: 5'-CTGTACTTTGTCAAGGT GGATGTGA-3' 5'-GTACGGCTGGAGGTCTGTCAAG-3'; 5'-CAGGCTCACGGAGGTCATCGCC-3', the amplicon length is 198 bp. Forward, reverse primers and probe for the reference gene *ACTB* (#NM_001101.2): 5'-GTGCTCA GGGCTTCTTGTCCTTT-3', 5'-TTTCTCCATGTCGT CCCAGTTGGT-3', 5'-AGGATTCCTATGTGGGCGA CGAGGCCCA-3', the amplicon length is 160 bp. Forward, reverse primers and probe for the reference gene *GUSB* (#NM_000181): 5'-TGCCGTGAGTCTCTGCTGTG-3', 5'-CCTACGCACCACTTCTTCCATC-3', 5'-TGACCCTC TGTCCCTTCCCTCCTG-3', the amplicon length is 152 bp. Forward, reverse primers and probes for the *GAPDH* (# NM_002046): 5'-CGGAGTAACGGATTTGGTC-3', 5'-TG GGTGGAATCATATTGGAACAT-3', 5'-CCCTTCATT GACCTCAACTACATGGTTTACAT-3', the amplicon length is 141 bp. All probes were labeled with a reporter dye (6-carboxy-florescein, FAM) at their 5'-end and a dark quencher dye RTQ1 (Sintol, Russia) at their 3'-end.

Quantification of hTERT mRNA level in SCC. TaqMan qPCR was carried out with Sequence Detection System ABI 7000 PRISM™ SDS (Applied Biosystems). For qPCR mixtures the following final compositions were used: 12.5 μl standard 1X qPCR buffer Reality (EIMB RAS, Moscow) (9), the analogue of TaqMan Universal PCR Master Mix (Applied Biosystems), primers and probes in optimal concentrations and 2 μl template in total volume of 25 μl in triplicate. Final concentrations were as follows: *hTERT* primers - 300 nM, probe - 300 nM (set 1) and primers - 300 nM, probe - 400 nM (set 2); *GAPDH* primers - 300 nM, probe - 150 nM. The thermocycler conditions were 10 min at 95˚C, then 50 two-step cycles 15 sec at 95˚C and 60 sec at 60˚C. The sequences of the amplicons were verified by sequencing in a 3730 DNA Analyzer automated sequencer (Applied Biosystems). qPCR data were analyzed using the relative quantification (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf) based on mRNA levels ratio of *hTERT* gene normalized to a reference gene *GAPDH* in a given tumor sample relative to another reference sample. All necessary control experiments were done. We performed standardization of all assays, selection of reference samples, evaluation of reference genes variability and efficiency of PCR reactions (http//www.gene-quantification.info/). As a reference samples we used two tumor cDNA samples with minimal *hTERT* expression instead of normal cDNAs where no *hTERT* transcripts were detected. The variability of *GAPDH* mRNA level was no higher than 2-fold in tumor (T) and normal (N) tissues. The raw data were analyzed using the program AEGIS (analysis of expression of genes in paired samples, the registration number 2006613816, 2006, Rospatent, Russia). The reaction efficiencies (E) calculated using the AEGIS and relative standard curves (*) were similar: E = (80±7)%, *E = (75±7)% for *hTERT* in tumors; E = (89±8)%, *E = (92±9)% for *GAPDH* in T and N. All analyzed tumors expressed n-fold amount of *hTERT* mRNA relative to the reference samples. For 3 amplification curves only semiquantitative analysis was performed because of low E values for *hTERT* in SCC samples.

Quantification of hTERT DNA copy number in SCC. Final concentrations were for *hTERT* primers - 300 nM, probe - 300 nM; *ACTB* primers - 200 nM, probe - 100 nM; *GUSB* primers - 200 nM, probe - 200 nM. Other conditions were the same as for mRNA quantification. As a reference we used paired normal samples or their pools. All analyzed tumors expressed n-fold copy number of *hTERT* DNA relative to the reference samples. Values E in normal and tumor samples were close to each other: (92±8)% for *hTERT*; (82±6)% for *ACTB* and (86±4)% for *GUSB*. The variability of *ACTB* and *GUSB* copy number was no higher than 1.6-fold in tumor (T) and normal (N) tissues.

Statistical analysis. We compared mRNA level and DNA copy number differences of target and reference genes for the same cervical cancer patients using a non-parametric Wilcoxon test. The evaluation of statistical significance of

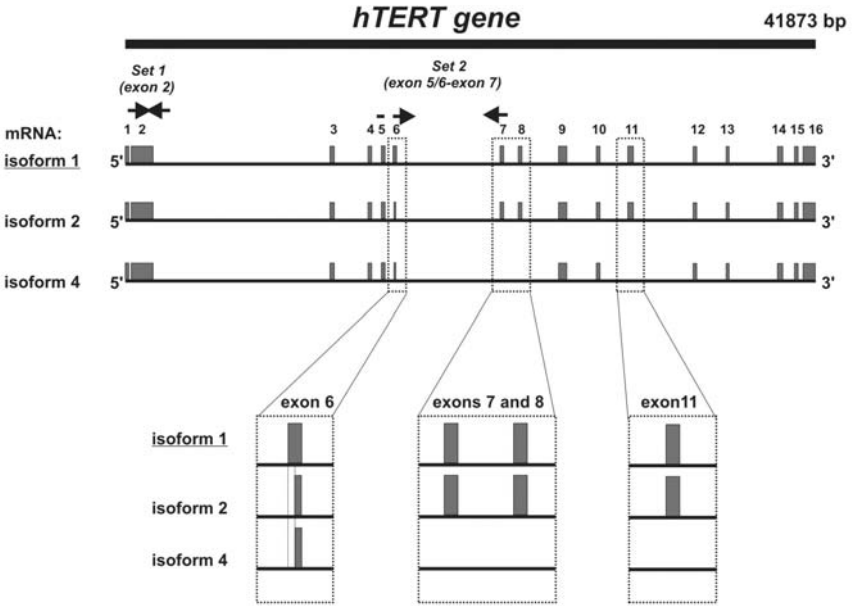

Figure 1. Schematical map of the *hTERT* and mRNA isoforms. Primer positions are designated by arrowheads. Structure of the *hTERT* full-length isoform 1 and non-functional isoforms 2 and 4 is also shown.

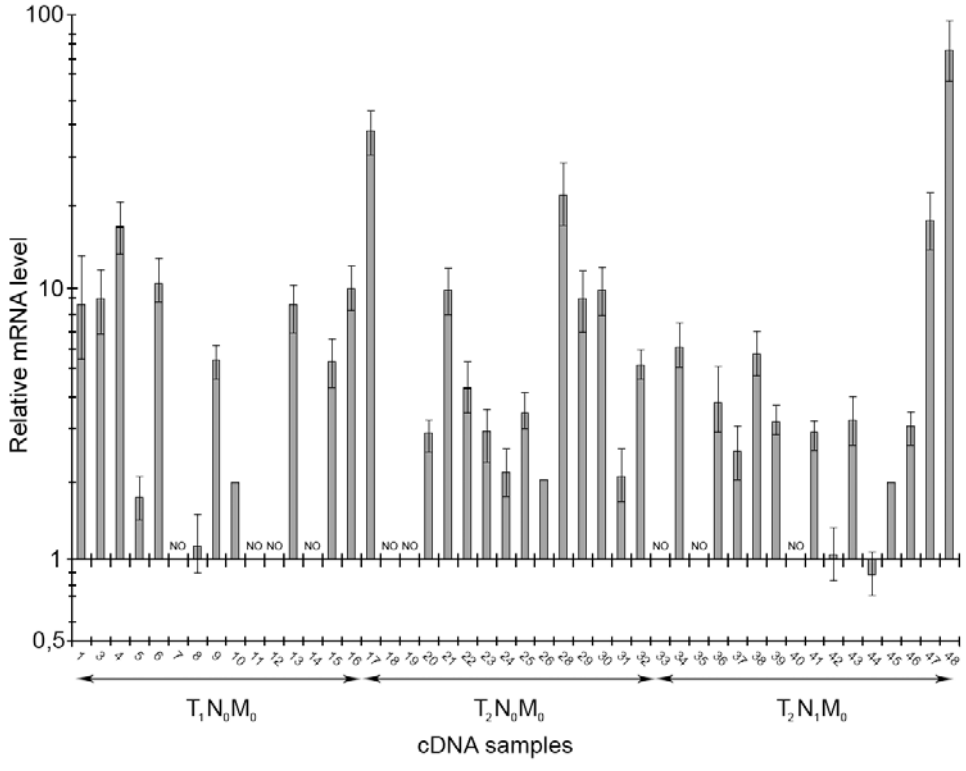

Figure 2. The level of *hTERT* mRNA in primary SCC relative to the reference gene GAPDH.

mRNA level was tested for all studied cases and for paired groups with different clinical characreristics. P-values <0.05 were considered statistically significant. The statistical analysis was performed using the BioStat software 'Practice' (2006) as previously described (10).

Results

Three variants of *hTERT* gene transcripts were known: the full-size isoform 1 (Genbank #NM_198253); isoform 2 encoding a non-active protein (#NM_198255), and truncated isoform 4 that does not encode any protein (#NM_198254). Isoform 1 differs from isoforms 2 and 4 by localization of exon-exon borders. In order to quantify *hTERT* mRNA in SCC we designed two primer sets (set 1 and set 2) that distinguish the full-length isoform 1, isoform 2 and isoform 4 (Fig. 1). We used qPCR for *hTERT* mRNA level and gene copy number analysis in SCC and adjacent normal cervical tissues. To examine *hTERT* expression we applied primer set 2. In 80% (37/46, p<0.001) of SCC *hTERT* gene expression was

Table I. The *hTERT* mRNA level and *hTERT* copy number in SCC.

TNM classification	Frequency increase %	
	mRNA level	DNA copy number
	Increase	Retention
$T_1N_0M_0$	75 (12/16) p<0.02	100 (15/15) p<0.01
$T_2N_0M_0$	81 (13/16) p<0.02	100 (11/11) p<0.01
$T_1N_0M_0 + T_2N_0M_0$ without metastases	78 (25/32) p<0.001	100 (26/26) p<0.01
$T_2N_1M_0$ with metastases	86 (12/14) p<0.02	100 (7/7) p<0.01
Total	**80** (37/46) p<0.001	**100** (33/33) p<0.01

Table II. Relative *hTERT* mRNAs level in SCC for two sets of primers and probes.

Sample no.	TNM classification	Relative *hTERT* mRNAs level (n-fold + range)	
		Set 1	Set 2
1	$T_{1b}N_0M_0$	10 (9-11)	9 (6-13)
5	$T_{1b}N_0M_0$	≤10	10 (7-12)
10	$T_{1b}N_0M_0$	No	No
13	$T_{1b}N_0M_0$	No	No
14	$T_{1b}N_0M_0$	6.0 (5-7)	5 (4-6)
15	$T_{1b}N_0M_0$	14 (12-16)	10 (8-12)
16	$T_{1b}N_0M_0$	27 (24-30)	36 (29-45)
17	$T_{2b}N_0M_0$	No	No
21	$T_{2a}N_0M_0$	≤8	5 (4-6)
22	$T_{2b}N_0M_0$	≤4	3 (2-5)
23	$T_{2a}N_0M_0$	≤2	2.1 (1.7-2.6)
26	$T_{2a}N_0M_0$	9 (8-10)	22 (17-29)
27	$T_{2a}N_0M_0$	7 (6-8)	9 (7-11)
32	$T_{2a}N_0M_0$	3 (2-4)	5.5 (4-7)
37	$T_{2b}N_1M_0$	≤4	3 (2-4)
38	$T_{2b}N_1M_0$	No	No
39	$T_{2b}N_1M_0$	≤1.5	2.5 (2-3)
40	$T_{2b}N_1M_0$	≤2	1.0 (0.8-1.3)
43	$T_{2b}N_1M_0$	No	≤2
45	$T_2N_1M_0$	13 (11-15)	18 (14-23)
46	$T_{2b}N_1M_0$	91 (86-95)	80 (72-95)

activated in tumor tissues (Fig. 2). The range of activation was between 2- and 80-fold. In 35% (16/46) of cases expression increased from 2- to 10-fold, and in 24% (11/46) of cases >10-fold increase was observed. Maximal increase was 80-fold (range 72-95) for a single specimen No 46 ($T_2N_1M_0$). The frequency and the extent of the increase of *hTERT* mRNA was independent of tumor size (T_1-T_2) and of the presence of metastases in regional lymph nodes (N_1) (Fig. 2, Table I). In 20% (9/46) of cases expression of *hTERT* was below detectable level.

To define if the detected *hTERT* overexpression in SCC was linked to functionally active full-lenghth isoform 1 of telomerase, we compared the results of mRNA quantification using primer sets 1 and 2 only for 21 RNA samples because of limited quantity of template. For 91% (19/21) of tested samples the data were similar for both sets (Table II). Thus, *hTERT* mRNA was synthesized as a full-length isoform 1.

In order to answer the question, whether the increase of *hTERT* mRNA level resulted from gene amplification, we performed quantitative evaluation of *hTERT* gene copy number using primer set 1 (Fig. 3, Table I). The results were normalized vs. two reference genes. The average *hTERT* DNA copy number was equal to 1.1±0.2 for both *ACTB* and *GUSB* genes. Finally, copy number of the *hTERT* was virtually unchanged in all 33 tumor samples compared to adjacent normal tissues, independently of the tumor size (T_1-T_2) and the presence of metastases in regional lymph nodes (N_1). The data that we obtained for primary tumors were concordant with those for the five cell lines (Fig. 4).

Discussion

Previously, qPCR was not applied to study *hTERT* expression and copy number in SCC. The *hTERT* mRNA level was analyzed by RT-PCR at different stages of cervical squamous intraepithelium lesions. A significant increase in mRNA content was found in early and late dysplasias, 35-40% and 89-96%, respectively (6,11). In our study, the increase of *hTERT* mRNA was found in 80% of all SCC samples and in 75% of cases at the initial stages of tumor development ($T_1N_0M_0$). This increase was virtually independent from tumor size (T_1-T_2) and from the presence of lymph node metastases. In this study, we also demonstrated that *hTERT* mRNA in SCC was synthesized in the form of functionally active full-length transcript. Increased level of full-size mRNA is in agreement with our previous data on detecting telomerase activity in SCC (6). Recently, increased *hTERT* protein expression (including early stages) was shown by immunohistochemical method (13). The absence of *hTERT* mRNA in 20% of cases in our experiments may be associated with possible switch to the alternative telomerase-independent mechanism of telomere elongation (14,15), though this assumption requires experimental verification.

The mechanism of *hTERT* gene activation still remains unclear. Earlier, *hTERT* gene amplification in SCC was demonstrated by FISH in cell lines and in primary tumors (4,5). In our study, qPCR data showed that *hTERT* was not amplified in all 33 SCC samples tested so far. It cannot be explained by inappropriate quality of DNA because in the

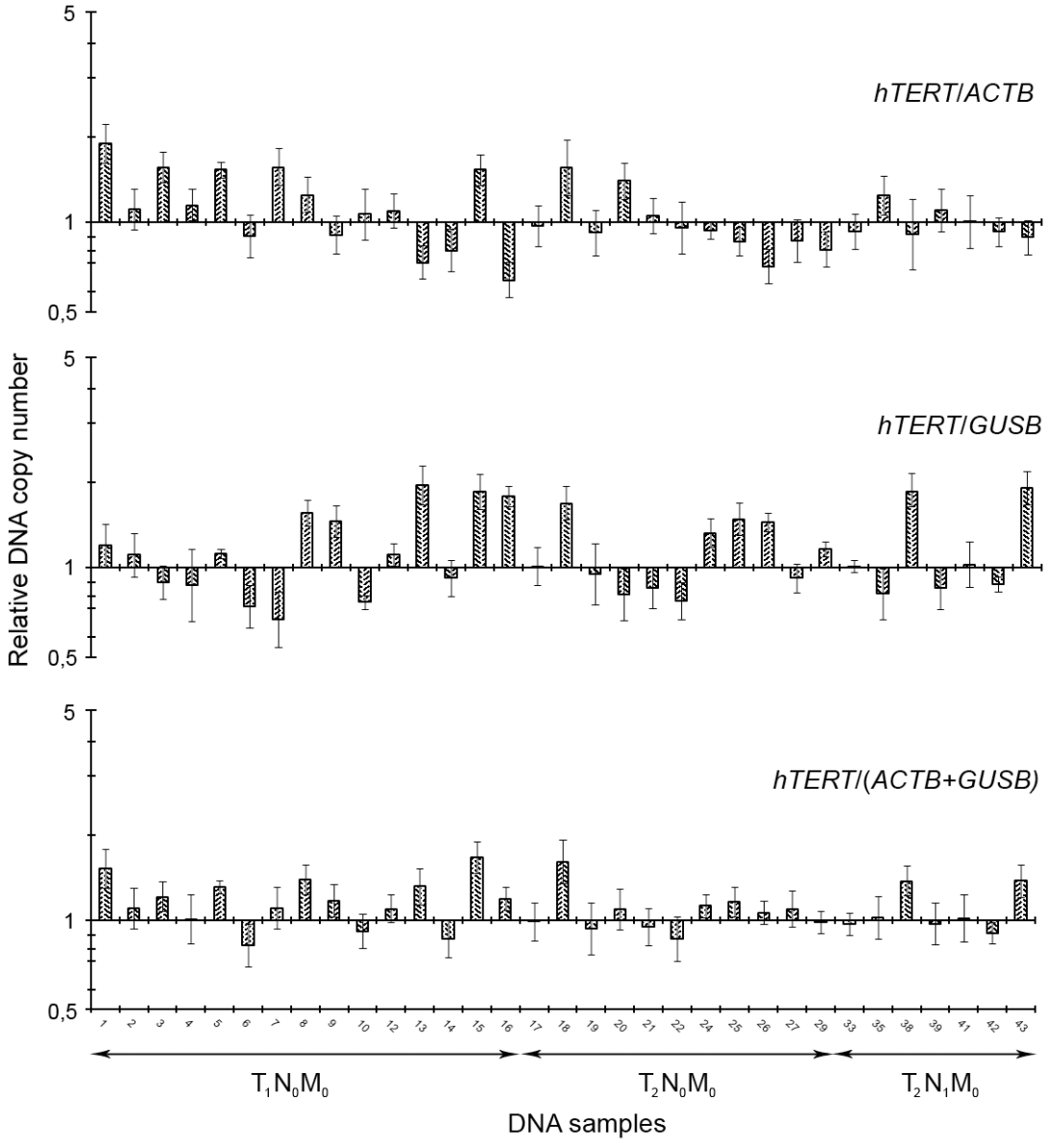

Figure 3. The *hTERT* DNA gene copy number in primary SCC relative to the reference genes *ACTB*, *GUSB* and the two genes together.

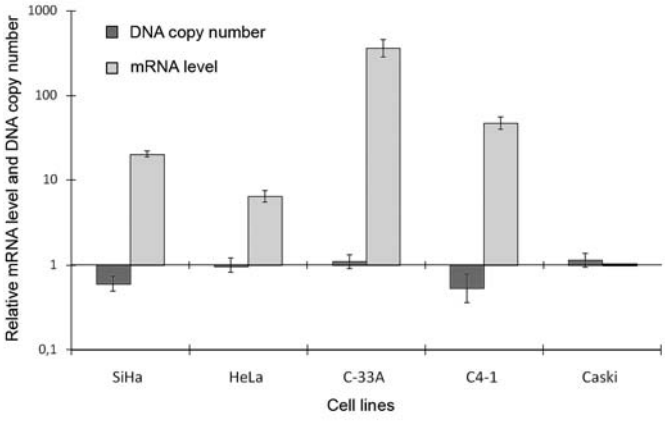

Figure 4. The quantitative evaluation of *hTERT* mRNA level and gene copy number in five cell lines. *hTERT* level is presented in comparison to CaSki cells, used as reference sample due to their minimal level of *hTERT* mRNA.

same samples we found earlier that *CTDSPL/RBSP3* gene was deleted in 42% (19/45) and amplified in 9% (4/45) (8). Thus, *hTERT* transcription was activated in SCC independently of gene amplification. This effect could be caused by promoter demethylation and/or direct effect of HPV-encoded viral proteins. Methylation of the *hTERT* gene promoter was found in primary tumors and cell lines, but no correlation was found between methylation and *hTERT* mRNA level (16,17). Possible role of proteins encoded by high risk HPV was studied and activation of *hTERT* transcription by HPV 16 E6 protein was demonstrated in human keratinocytes (18). In primary SCC, the positive correlation was found between viral load with oncogenic HPV and telomerase activity (19). Although *hTERT* amplification was detected in SCC (4,5) and other cancers (20), several studies presented evidence of transcriptional (16,17,21) and post-transcriptional (22) mechanisms of *hTERT* expression activation. These data agree with amplification-independent activation of *hTERT*

expression observed in our study. Previously detected *hTERT* gene rearrangements could be a side effect of overall cancer genome instability, not related to activation of *hTERT* transcription.

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